Evaluation of antioxidant and anticancer efficacy of chitosan based nanoparticles

Thesis submitted to
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BY:
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CERTIFICATE

This is to certify that the thesis entitled "***Evaluation of antioxidant and anticancer efficacy of chitosan based nanoparticles***" Submitted to National Institute of Technology, Rourkela for the partial fulfillment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by **Ms. Aliva Prity Minz** under my supervision and guidance. The results embodied in this thesis are new and have not been submitted to any other university or institution for award of any degree or diploma.

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DECLARATION

I hereby declare the thesis entitled **"*Evaluation of antioxidant and anticancer efficacy of chitosan based Nanoparticles*"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide research work carried out by me under the guidance and supervision of Dr. BismitaNayak, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date: Place: AlivaPrityMinz

Place:

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Abstract

Synthesis of chitosan based nanoparticle is a novel way for synthesis of nanoparticles by using biological sources. It is gaining attention due to its cost effective, ecofriendly and large scale production possibilities. In the present study six formulations are made taking chitosan as base material: chitosan-ascorbic acid(Cs-Aa), chitosan-ascorbic acid-Glucose(Cs-Aa-Glu), chitosan-Polyethyl glycol (Cs-PEG), chitosan-vitamin E (Cs-Vitamin E),chitosan-catechol (Cs-Cat),chitosan-Silver nanoparticles (Cs-AgNp)were taken to investigate their antioxidant property. The appearance of milky white solution indicates that nanoparticles are being formed. The characterization studied was done by UV-Visible spectroscopy, Scanning Electron Microscopy (SEM), Dynamic Light Scattering (DLS) and zeta potential studies, X-Ray diffraction (XRD), Fourier Transmission Infrared Spectroscopy (FTIR).

Keywords- Nanoparticles, SEM, AFM, FTIR, DLS, XRD

INTRODUCTION

The term “nano” is derived from the Greek word meaning “dwarf” [1]. Nanoparticle ranges in size from 100 nm - 1 nm. Today this field has ventured beyond technology into physics, chemistry and biology creating myriad opportunities for advancing medical science. Nanotechnology is useful in research and development of almost every economic sector, from aerospace to medicine to energy[2] such as drug development,water decontamination,information and communication technology etc.

The structural and functional unit of nanotechnology is nanoparticle. The major issue in biomedical application is drug delivery to the target organ which is an emerging concern in biomedical sciences because 90% of all potential therapeutics has poor pharmacokinetics and biopharmaceutical properties.Nanoparticles can be efficiently used for drug delivery to desired location/organ.

Nanoparticles are miniature entity that behaves as a whole unit with respect to its transport and property.Various types of nanoparticles can be synthesized like liposome, nanosphere, nanocapsule, dendrimer, polymeric micelles and SLN (solid lipid nanoparticles). Liposomes are lipid vesicle formed by hydration which encloses the hydrophilic (water soluble) drugs like doxorubicin,mitoxantrone within itself[3].

Recently, Solid Lipid Nanoparticles (SLN) has blossomed as novel carrier systems for cosmetic and biomedical drugs [4, 5]. SLN are formed by a matrix of lipids which are biodegradable raw materials that are physiologically well tolerated[6]. The main advantages of these systems include protection of suitable substances from degradation within our cell and controlled release of substances due to the solid state of the lipid matrix[7]. Even though SLN are compounded by physiological ingredients and can be easily produced, they present some disadvantages such as low drug-loading capacities.

The use of polymeric materials for encapsulating drugs or other active substances is an important approach to mask the physico-chemical intrinsic properties of substances facilitating their skin

penetration [8]. Apart from this, these nano-medicines are stable in blood, non-toxic, non-thrombogenic,non-immunogenic, non-inflammatory, do not activate neutrophils,biodegradable, avoid macrophage system and applicable to various molecules such as drugs, proteins, peptides, or nucleic acids[9]. Most commonly used biodegradable polymers are poly (D, L-lactide), poly(lactic acid) (PLA) or poly (lactide-co-glycolide), poly-alkyl-cyanoacrylates, chitosan and gelatin.

Chitosan

Chitosan (CS) is a natural polysaccharide modified from chitin which is extracted from crustaceans. In other word the deacetylated form of chitin is called chitosan. Four methods are reported for the preparation of chitosan nanoparticles, these include ionotropic gelation,microemulsion, emulsification solvent diffusion and polyelectrolyte complex. Ionotropic gelation is based on electrostatic interaction between amine group of chitosan and negatively charged groups of a polyanion such as tripolyphosphate (TPP). Chitosan is dissolved in acetic acid. Polyanion was then addeddropwiseand nanoparticles were formed spontaneously under mechanical stirring.

Reactive Oxygen Species

Reactive Oxygen Species (ROS) are unstable and reactive molecules containing oxygen. Examples include oxygen ions and peroxides. ROS are natural byproduct of the normal metabolism of oxygen and plays an important role in cell signaling and homeostasis.However, during environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically. This may result in significant damage to cell structures. This phenomenon is known as oxidative stress. Oxidative stress reflects an imbalance between the reactive oxygen species and a biological system's ability to readily detoxify the reactive mechanism. Oxidative stress may lead to damage of proteins, lipids, and DNA. In humans, oxidative stress is thought to be involved in the development of cancer [10], Parkinson's

disease, Alzheimer's disease,atherosclerosis, heart failure,myocardial infarction [11]. This oxidative stress can be decreased by antioxidants.

Antioxidants

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. Antioxidants are often reducing agents such as ascorbic acid, vitamin C,vitamin E, etc.

Vitamin E

Vitamin E is a fat-soluble antioxidant, it stops the production of reactive oxygen species formed when fat undergoes oxidation[12-13]. It performs its functions as antioxidant in the glutathione peroxidase pathway[14],and it protects cell membrane from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction.Chronic pharmacological treatment with alpha-Tocopherol reversed pathophysiological hallmarks including free radical overproduction, oxidative stress[15].

PEG- When attached to various protein medications, polyethylene glycol allows a slowed clearance of the carried protein from the blood. This makes for a longer-acting medicinal effect and reduces toxicity, and allows longer dosing intervals. PEG chains attached to a surface or forming the corona of a nanosphere exhibit rapid chain motion in an aqueous medium and have a large excluded volume[16].

The most common antioxidants with proven ROS quenching activity are VitaminE,Catechol,and Ascorbic acid.

In this work we have tried to synthesize chitosan nanoparticle conjugates to address the various issues like oxidative stress,better permeability into the target cell,better stability etc.

REVIEW OF LITERATURE

Nanotechnology is the term given to those areas of science and engineering in which materials are designed at nanometer range.In broad sense it can be defined as to designing, characterizing, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nanometer scale with at least one novel/superior characteristic or property [17].

At the nanoscale, cells record information, process information, carry out a set of instructions, transform energy from one form to another, replicate themselves, and adapt to changing environments in ways that allow best possible performance for necessary tasks. Biological systems provide great inspiration for the design of functional nanoscale structures and can also help us learn how to organize nanostructures into much larger systems.

Nanotechnology field seeks to realize how the structure of materials is connected to their properties and thereby enable "materials by design," with special properties required for particular applications. It is within this range(1-100nm) that materials can have substantially different properties compared to the same substances at larger sizes, both because of the substantially increased ratio of surface area to mass, and also because quantum effects begin to play a role at these dimensions, leading to significant changes in several types of physical property.Nanoparticles as medication conveyance frameworks empower exceptional methodologies for malignancy treatment. In the course of the most recent two decades, countless conveyance frameworks have been produced for malignancy treatment, including natural and inorganic materials.

Nanomaterials are excellent agents for filters in liquid separations of various sorts, for example, for purification of waste water nanoscale titania and zirconia materials can trap heavy metals and bio-organism.

Nanoparticles have already been used in timed-release drug delivery systems. Nanoparticles with their special characteristics small particle size, large surface area and the capability of changing their surface properties have numerous advantages compared with other delivery systems.

Numerous liposomal, polymer-medication conjugates, and micellar plans are a piece of the best in class in the facilities, and a considerably more noteworthy number of nanoparticle stages are at present in the preclinical phases of advancement. In the course of recent decades, noteworthy advancement has been made in the improvement and use of built nanoparticles to treat tumor all the more viably. For instance, helpful specialists have been coordinated with nanoparticles built with ideal sizes, shapes, and surface properties to expand their dissolvability, delay their course half-life, enhance their bio-distribution, and decrease their immunogenicity. Nanoparticles and their payloads have likewise been positively conveyed into tumors by exploiting the pathophysiological conditions, for example, the upgraded penetrability and maintenance impact, and the spatial varieties in the pH esteem. Also, focusing on ligands (e.g., little natural atoms, peptides, antibodies, and nucleic acids) have been added to the surface of nanoparticles to explicitly target malignant cells through specific tying to the receptors overexpressed on their surface.

2.1.The advantages of using Nanoparticles as a drug delivery system include the following:

1. Nanoparticles are small molecule that acts as a stable molecule with respect to its transport and maintain its properties.
2. Particle size and surface characteristic of Nanoparticles can be easily modified to achieve drug delivery of our need.
3. Nanoparticles control the release of the drug during the transportation and at desired site.
4. Site-specific targeting can be achieved by attaching specific ligands to surface of particles or use of magnetic guidance.
5. The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular etc. [18].
6. Nanoparticles can penetrate to tiny areas within the body.
7. Usage of effective and efficient drug carriers reduces drug toxicity and provides adequate drug delivery.
8. Nanocarriers can deliver drugs to anatomical barrier body parts such as blood brain barrier.
9. Chitosan have low toxicity, better stability and biodegradability and can be administered through oral, nasal and other routes[19].

2.2.CHITOSAN

Chitin is a white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas. Chitosan is the *N*-deacetylated derivative of chitin, although this *N*-deacetylation is almost never complete [20].Commercially available CS has an average molecular weight ranging between 3800 and 20,000 Daltons and is 66% to 95% deacetylated. The chitin is

deacetylated in 40% sodium hydroxide at 120°C for 1–3 h. This treatment produces 70% deacetylated chitosan. The size of nanoparticle can be controlled by playing with the deacetylation process. These are used to make nanoparticles and micro particles, nanoparticles ranges insize from 1 to 200 nm and micro particlespossess a size of 1–1000 μm [21].

Chitosan exists in cationic form in neutral or basic solution therefore insoluble in water,in contrast at acidic pH it gets protonated due to free distribution of amino and N-acetyl groups thus being soluble in water. Usually 1-3% of acetic acid is used to dissolve chitosan but in our formulation we take 1% of ascorbic acid to dissolve chitosan.

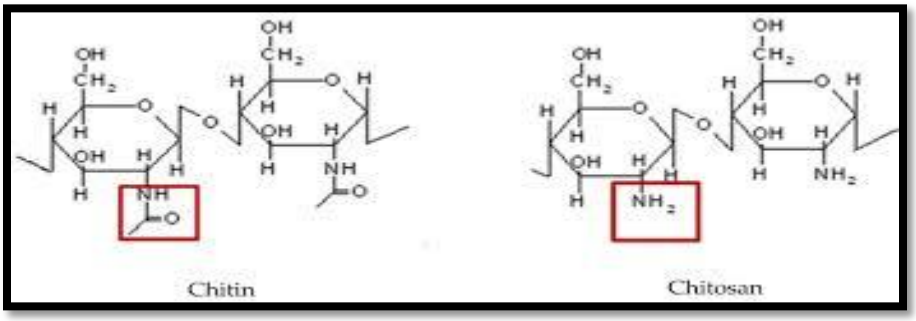


Figure2.2a. Molecular structure of chitin and chitosan

Chitosan gets easily degraded by living cells and easily removed out of body without side effect.Chitosan is considered the most important polysaccharide for various drug delivery purposes because of its cationic character and primary amino groups, which are responsible for its many biological properties such as mucoadhesion,controlled drug release, transfection, in situ gelation, and efflux pump inhibitory properties and permeation enhancement.

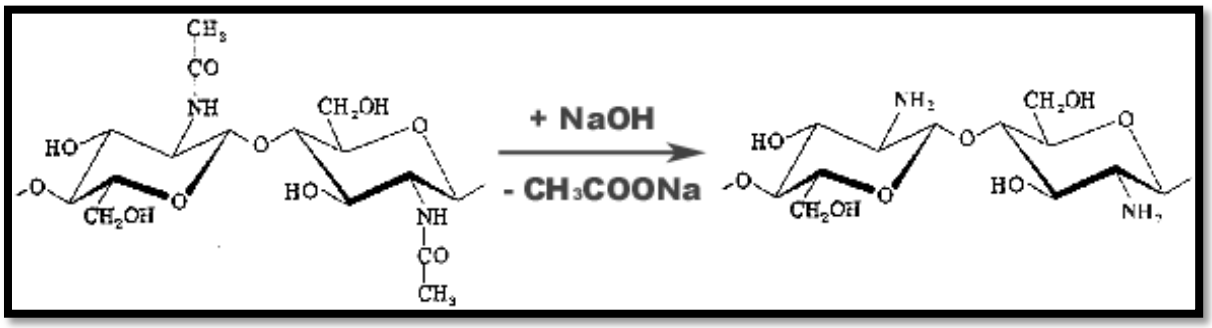


Figure 2.2b: Chemical reaction showing deacetylation of chitin to form chitosan

2.3.Types of Nanoparticles

2.3.1.Liposomes

Liposomes are artificially prepared bilayered vesicles made of lipid bilayer in which an aqueous volume is entirely enclosedthe aqueous volume include the nutrients and pharmaceutical drugs. Liposomes are definite as spherical vesicles with particle sizes ranging from 30 nm to several micrometers. Advantage of liposome is that these show amphiphilic character, biocompatibility,andmodifying the surface these drugs can be suitable candidate for delivery of biotech drugs, proteins and peptides to desired site. Liposomes have increased circulation half-life.

The main disadvantage of the standard liposome formulations includes their rapid clearance from circulation due to uptakeby the ReticuloEndothelial System(RES), primarily in the liver.To circumvent this problem,long-circulating liposomes with Polyethylene Glycol (PEG) molecules attached to their surfaces have been developed. The PEG molecule forms a protective layer over the surface,reducing opsonin binding to liposomes, thus prolonging their serum half-life.

However, developmental work on liposomes has been limited due to inherent problems such as low encapsulation efficiency, rapid leakage of water-soluble drug in the presence of blood components and poor storage stability [22].

2.3.2.Nanocrystals and Nanosuspension

Nanocrystals are nanoparticles that have crystalline structure with size of nanorange that is 1000nm. A nanosuspension indicates the drug nanocrystals dispersed in stabilizing agents such as surfactants,water or nonaqueous solution [23].Nanocrystals have many advantages i.e. its bioavailability and good absorption characteristics.Nanocrystals can be used for targeting the mucosa, cells of the mononuclear phagocytic system (MPS) to treat infections of the MPS from mycobacterial infections and leishmaniasis. These nanocrystals serve as delivery system for drugs like amphotericin B, tacrolimus, etc. The size ofnanocrystals allows it to pass effectively through capillaries.

2.3.3.Solid Lipid Nanoparticles

Solid Lipid Nanoparticles (SLN) are aqueous colloidal dispersions, the matrix of which comprises of solid biodegradable lipids [24]. SLN provides site specific delivery of drugs, enhanced drug penetration into the skin via dermal application. It improves bioavailability of poorly water soluble molecules [25]. Using a biodegradable physiological lipid decreases the danger of acute and chronic toxicity and avoidance of organic solvents in production methods [26]. SLNs have better stability compared to liposomes [27]. Disadvantage of SLNis that it provides poor drug loading capacity.

2.3.4.Polymeric Nanoparticles:

Polymeric nanoparticles (PNPs) consist of a biodegradable polymer. Biocompatibility is anessential feature for potential application as tissue engineering, drug and gene delivery and newvaccination strategies. Latest developments also include natural polymers like chitosan, gelatin, andsodium alginate to overcome some toxicological problems with the synthetic polymers. Polymericnanoparticles represent a significant improvement over traditional oral and intravenous methods ofadministration in terms of efficiency and effectiveness. Polymeric Nanoparticles are non-immunogenic, non-inflammatory, do not activate neutrophils, biodegradable, avoid Reticulo Endothelial System (RES) and applicable to various molecules such as drugs, proteins, peptides, or nucleic acidsApart from this, these nano-medicines are stable in blood, non-toxic, non-thrombogenic.

2.3.5.Dendrimers

The well-defined structure, mono-dispersity of size, surface functionalization capability, and stability are properties of dendrimers that make them attractive drug carrier candidates.Drug molecules can be incorporated into dendrimers via either complexation or encapsulation.

2.3.5a.Poly-d,l-lactide-co-glycolide (PLGA)

Poly(lactic-co-glycolic acid) (PLGA) is one of the most successfully used biodegradable polymers because its hydrolysis leads to metabolite monomers, lactic acid and glycolic acid. Because these two monomers are endogenous and easily metabolized by the body *via* the Krebs cycle, a minimal systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications [28], biodegradability and biocompatibility, protection of drug from degradation

2.3.5b.Poly (lactic acid) (PLA)

Poly(lactic acid) (PLA) has also been used to a lesser extent than PLGA due to the lower degradation rate [29]. PLA polymer is biocompatible and biodegradable that undergoes degradation in our body to monomeric units of lactic acid which is utilized in carbohydrate metabolism.

2.3.6.Hydrogels

Hydrophilic gels that are usually referred to as hydrogels are networks of polymer chains that are sometimes found as colloidal gels in which water is the dispersion medium [30], another definition is that it is a polymeric material that exhibits the ability to swell and retain a significant fraction of water within its structure, but will not dissolve in water. The hydrogel technologies may be applied to, drug delivery systems and biomedical applications and tissue engineering and regenerative medicines and diagnostics, wound dressing, separation of biomolecules or cells and barrier materials to regulate biological adhesions.

2.4.Antioxidants used

2.4.1.Vitamin E

Vitamin E, a potent peroxyl radical scavenger, is a chain-breaking antioxidant that prevents the damage of biological membrane from free radical. Vitamin Eis the primary lipid soluble antioxidant, which may have an important role in scavenging free oxygen radicals and in stabilizing the cell membranes, thereby maintaining its permeability.Moreover, it is known that antioxidants, such as Vit E, coenzyme Q, vitamin C, Glutathione (GSH) and selenium may act synergically, preventing lipid peroxidation and cell destruction.

2.4.2.Catechol

To facilitate the discovery of antioxidants, some antioxidant pharmacophores have been identified, among which catechol has been given the most attention [31][32][33].The great antioxidant potential of catechol is attributed to the fact that the semiquinone radical derived from H-atom donation of catechol can be stabilized by an intramolecular hydrogen bond and the electron-donating properties of the *ortho*-OH .Although much effort has been devoted to designing novel antioxidants based on catechol, thepotential toxicity of catecholics is a big concern, because the catechol-derived semiquinone radical canstill donate a H-atom to generate quinone, and during this process a superoxide anion radical may be produced [34-35-36] In addition, quinones may act as Michael acceptors to react with various nucleophilicbiomolecules producing cytotoxicity.

2.4.3.Ascorbic Acid

Ascorbic acid or Ascorbate has been demonstrated to be an effective antioxidant. It can act both directly, by reaction with aqueous peroxyl radicals, and indirectly, by restoring the antioxidant properties of fat-soluble vitamin E. The overall consequence of these antioxidant activities is the beneficial control of lipid peroxidation of cellular membranes including those surrounding as well as within intracellular organelles. Intracellular free radical attack on non-lipid nuclear material may also be diminished. Two human breast carcinoma cell lines, MCF-7 and MDA-MB-231, were tested against doxorubicin (DOX), cisplatin (DDP), and paclitaxel (Tx) alone and in combination with ascorbic acid (Vitamin C). In both cell lines, Vitamin C exhibited cytotoxic activity at high concentrations (i.e. 10^2–10^3μM) [37].

Both cell lines were strongly sensitive to paclitaxel and Vitamin C both at non-cytotoxic (1 μM) and moderately cytotoxic concentrations (10^2μM) improved the cytotoxicity of DOX, DDP, and Tx significantly. Combination effects between Vitamin C and DDP or Tx were partly synergistic and partly additive or sub additive whereas a consistent synergism was found between Vit C and DOX. The mechanisms by which Vitamin C potentiates the cytostatics studied are yet unclear and need to be evaluated further.

2.5.Glucose

Experiments by Cori and Cori (1925) and by Warburg et al. (1927) showed that tumors appeared to be avidly consuming glucose and producing La^- [38-39]. Malignant cells have accelerated metabolism and increased requirements for ATP production.It is known that the cancer cells have high rates of aerobic glycolysis. Aerobic glycolysis can result in accumulation of lactate in cancer cells, due to the elevated levels of lactate dehydrogenase converting pyruvate to lactate [40].

This means in cancer cells the membrane bound Glucose Transporters (GLUTs) are highly active than normal cells, thus Gluts be identified as markers for early detection and prognosis of carcinoma. GLUTs area family of five functional members i.e., GLUT 1, GLUT 2, GLUT 3, GLUT 4 and GLUT 5 where the expression of GLUT 1, 2, 4 and 5 have been reported to increase in case of MDA-MB-468 and MCF-7 breast cancer cell lines. GLUT12 was discovered in a human breast cancer cell line and is also expressed in human breast and prostate cancer, the rat lactating mammary gland and a wide range of tissues in the rat fetus, all tissues with high rates of glucose utilization. Recently, Macheda et al., 2002, have shown that GLUT12 protein is expressed in the breast cancer cell line MCF-7. The knowledge of glucose transporter has renewed much interest in the shrewd

relationship between glucose and cancer tissue where glucose-linked conjugates such as gluphosphamide are preferentially taken up by tumors.

MATERIALS AND METHODS

3.1. Preparation of antioxidant conjugated chitosan nanoparticles

The nanoparticles were prepared according to the procedure reported by Calvo et al., based on the ionic gelation of chitosan with TPP with slight modifications[41]. In brief, chitosan was dissolved in 1% ascorbic acid aqueous solutions with various concentrations (1, 1.5, 2, 2.5 W/V %) of glucose under constant stirring at room temperature. After complete dissolution of chitosan in ascorbic acid, TPP (5 ml) was added drop wise through a syringe at uniform stirring until the occurrence of milky white suspension. After 30 minutes of vigorous stirring the solution was centrifuged (C-24 BL, REMI, Mumbai, India) at 10,000 rpm for 30 minutes and the pellet obtained was washed thrice with distilled water. The nanoparticles were lyophilized and stored at 4°C until further used. Similarly,PEG, Vitamin E and catechol were added to the chitosan-ascorbic acid solution before the drop wise addition of TPP and the nanoparticles were lyophilized and stored.

3.2. Preparation of silver nanoparticle conjugated chitosan nanoparticles

Silver nanoparticles were synthesised by using the petals extracts of *hibiscus* reported by Nayak*et al*., [42]. The same protocol was followed to prepare silver nanoparticles conjugated chitosan nanoparticles with slight modifications. In the chitosan-ascorbic acid solution glucose with various concentrations (1, 1.5, 2, 2.5 W/V %) was added followed by PEG (v/v) and after its complete blending AgNPs (w/v) was added to the solution followed by drop wise addition of TPP under constant magnetic stirring condition. After vigorous stirring the AgNP-CS solution was centrifuged at 10,000 rpm for 30 minutes and the nanoparticles recovered were lyophilized and stored at 4°C for further use.

3.3. Characterization of the conjugated chitosan nanoparticles

3.3.1. Size and Zeta potential studies

The size (Hydrodynamic diameter), size distribution (Poly Dispersity Index) and zeta potential (Surface charge) of the conjugated nanoparticles were analyzed by Zeta sizer (ZS 90, Malvern Instruments Ltd, Malvern, UK). Prior to the measurement, formulations were diluted with PBS (pH 7.4) and the measurements were carried out in triplicate. Data acquired was through integrated Malvern Zetasizer Software.

3.3.2. Morphological studies by Fe-SEM

The morphology of the nanoparticles was investigated by Field emission-scanning electron microscopy (Nova NanoSEM 450/ FEI). In preparatory step, 100µl of the conjugated nanochitosan formulations were added onto a 10 mm glass slide and dried in vacuum dessicator overnight followed by storage at room temperature till FE-SEM analysis was performed. For analysis, the nanoparticles were fixed on adequate support and coated with gold using gold sputter module in a higher vacuum evaporator. Observations were taken under different magnifications performed at 15kv.

3.3.3. The Attenuated Total Reflection Fourier Transform Infrared spectroscopy (ATR- FTIR)

The Attenuated Total Reflection Fourier Transform Infrared (ATR- FTIR)spectroscopy analysis was conducted to corroborate the possible interaction of chitosan, ascorbic acid, vitamin E, TPP for the synthesis of nanoparticles. TheATR- FTIR was performed on a Bruker ALPHA spectrophotometer (Ettlinger, Germany) with a resolution of 4 cm^{-1}. The samples were scanned in the spectral region between 4000 and 500 cm^{-1} by taking an average of 25 scans per sample. 1 drop of sample was kept

on the sample holder and the samples were scanned and the result obtained was analyzed through OPUS software.

3.3.4. X-ray powder diffraction (XRD)

The X-ray powder diffraction (XRD) patterns of chitsan, ascorbic acid, vitamin E, TPP, silver nanoparticles and conjugated nanoparticles were obtained using X-ray diffractometer (PANalyticalX'Pert, Almelo, The Netherlands) equipped with Ni filter and Cu Kα (l = 1.54056 Å) radiation source. The diffraction angle was varied in the range of 10-80 degrees while the scanning rate was 5 degree/s.

3.4. Encapsulation efficiency (EE)

The EE of the conjugated chitosan nanoparticles were determined by the ultracentrifugation method reported by Ashe *et al*.[43]. Briefly, vitamin E, catechol, ascorbic acid and silver nanoparticles conjugated with the chitosan nanoparticles were separated from the non-conjugated chitosan nanoparticles by using ultracentrifugation at 30,000 rpm for 2 hours. The pellet obtained were re-dissolved in distilled water and the supernatant was also scanned with UV–Visible spectrophotometer [Lambda 35® (PerkinElmer, Waltham, MS, USA)] at a different wavelength specific for ascorbic acid (262), vitamin E (295), Catechol (500), Chitosan(201),Glucose(807) and silver nanoparticles (420) nm.

3.5. Effect of pH on size and charge

To know the effect of pH on the size and zeta potential of the prepared formulations the blank and conjugated chitosan nanoparticles were subjected to various pH conditions (2, 4, 6, 8, 10, and 12). The measurements were taken by Zeta sizer (ZS 90, Malvern Instruments Ltd, Malvern, UK). The

experiment was done in triplicates (n=3, mean± Stdev) and was plotted as a function of size and zeta potential.

3.6. In-vitro drug release study

The *in vitro* drug release profiles of the antioxidants (Vitamin E, catechol and ascorbic acid) and silver nanoparticles from the conjugated chitosan nanoparticles were investigated in phosphate buffer saline (pH 7.4) at 37°C. The conjugated chitosan nanoparticles were placed in a dialysis bag and dialyzed against 50 mL of PBS with constant shaking at 250 rpm for 12 hours. Aliquots (5ml) were periodically removed for the analysis of Glucose,PEG, Vitamin E, catechol, Ascorbic acid and silver nanoparticle .The volume of removed samples in PBS being replaced by fresh volume of PBS. The amount of PEG,Vitamin E, catechol, Ascorbic acid and silver nanoparticle was determined by monitoring the absorbance at their respective wavelength with UV–Visible spectrophotometer [Lambda 35® (PerkinElmer, Waltham, MS, USA)].

3.7. In vitro antioxidant activity:

3.7.1. DPPH scavenging assay

The free radical scavenging activity of the conjugated chitosan nanoparticles and the base components used (chitosan,PEG, Vitamin E, catechol, ascorbic acid, glucose and silver nanoparticles) were measured *in vitro* against the stable 2, 2, diphenyl-1-picryl hydrazyl (DPPH) according to the method described by Brand-William et al, 1995[44] with slight modifications. The stock solution was prepared by dissolving 4mg DPPH with 100 ml methanol and stored in dark at 20°C until required. The conjugate chitosan nanoparticles and the components alone with different concentrations (10-100µg/ml) were prepared in methanol. Ascorbic acid was used as standard. 1 ml of the methanolic plant extract was mixed with 1ml of DPPH solution and the reaction mixture was

shaken well and incubated in the dark for 30 minutes at room temperature. The Absorbance was measured at 517nm where lower absorbance indicated higher free radical scavenging activity. The scavenging activity against DPPH was calculated using the following equation:

Scavenging free radical activity (%) = [(Ac-As)/Ac] x100,

Where Ac was the absorbance of control (DPPH solution without the sample), As was the absorbance of DPPH solution in the presence of the sample (extract/standard).

3.7.2. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging activity of the methanolic plant extracts were determined according to the method of Ruch et al., with slight modifications. A solution of H_2O_2 (40mM) in Phosphate buffer saline (pH 7.4) was prepared. Different concentration (10-100µg/ml) of conjugated chitosan nanoparticles and the base components used (chitosan, Vitamin E, catechol, ascorbic acid, glucose and silver nanoparticles) (0.1ml) was added with 0.6 ml of H_2O_2 and the volume was made up to 3ml with PBS (pH 7.4). The blank solution contains only PBS and H_2O_2. After 30 minutes of incubation the absorbance was measured at 230 nm. The % scavenging activity of H_2O_2 was calculated using the equation:

% scavenging activity = [(Ac-As)/Ac] X 100

Where Ac is the absorption of the control and As is the absorption of the samples.

3.7.3. Ferrous ion chelating activity

The ferrous chelating activity of the conjugated chitosan nanoparticles and the base components used (chitosan, Vitamin E, catechol, ascorbic acid, glucose and silver nanoparticles) were estimated by the method of Dinis, Mandeira and Almeida (1994). 0.5 ml of each extract was added to 1.6 ml of

deionised water and to this 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 seconds 0.1ml of ferrozine (5mM) was added to the reaction mixture and incubated for 10 minutes at room temperature. The absorbance of the

Fe^{2+} and ferrozine reaction complex was measured at 562nm. The control contains all the reaction mixture except the plant extract. The fe^{2+} chelating activity was calculated by following the equation

$$\text{Chelating rate} = [(A_C - A_S)/A_C] \text{ X } 100\%$$

Where, A_C was the absorbance of the control and A_S was the absorbance in the presence of the sample extract.

3.7.4. Ferric reducing antioxidant power assay

The Ferric reducing antioxidant power assay (FARP) was measured by following the procedure of Oyaizu et al, 1986 with some modifications. Briefly, the reaction mixture consist of 1 ml of 1 % potassium ferricyanide and 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) in which 1 ml of conjugated chitosan nanoparticles were mixed in different concentrations and the reaction solution was incubated at 50°C for 20 minutes in a water bath. After incubation 2.5 ml of 10 % tricholoroacetic acid was added to each reaction mixture and centrifuged at 3000 rpm for 10 minutes. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml $FeCl_3$ (0.1%) the absorbance at 700 nm was measured after incubating the reaction mixture for 10 minutes. The blank contains all the reaction mixtures except 1% potassium ferricyanide.

3.7.5. Nitric oxide scavenging assay

Briefly, In 1 ml of 10mM sodium nitroprusside solution 1 ml of conjugated chitosan nanoparticles in different concentrations (10, 20, 40, 60, 80, 100 µg/ml) were mixed and incubated at 25° C for 150 minutes.1 ml of the incubated reaction mixture was taken out and mixed with 1 ml of Griess reagent

(1% sulphanilamide + 2 % ortho-phosphoric acid + 0.1% napthylethylenediamine) and the absorbance at 546 nm was measured after incubating the reaction mixture for 10 minutes.

3.8. Cell viability assay (MTT assay)

Briefly, MCF-7 cells were purchased from NCCS, Pune, India and were seeded in 96 well plates at the density of 3000 cells/well based on the doubling time in presence of 200µl MEM supplemented with 10% FBS and 1% penicillin-streptomycin solution and incubated for 24 hours in incubator containing 5% CO_2 at 37°C. After 24 hours of seeding, the existing media was removed and replaced by fresh media along with various concentrations of silver nanoparticles viz., 10, 20, 40, 60, 80, 100, 150 µg/ml and incubated for 48 hrs at 37°C, 5% CO_2. To detect the cell viability, MTT working solution was prepared from a stock solution of 5mg/ml in growth medium without FBS to the final concentration of 0.8 mg/ml.100 µl of MTT solution was added and incubated for 4 hours. After 4 hours of incubation, the MTT solution was discarded and 100 µl of DMSO solvent was added in each well under dark followed by an incubation of 15 minutes and the optical density of the formazan product was read at 595nm in a microplate reader (PerkinElmer, Waltham, MS, USA).

RESULTS AND DISCUSSIONS

4.1. Characterization of the prepared conjugated chitosan nanoparticles

Chitosan dissolved in ascorbic acid were entrapped by drop wise addition of tripolyphosphate (TPP). The method adopted is termed as ion gelation method. Generally 1% acetic acid is used to dissolve chitosan .but to enhance the antioxidant property we have replaced the acetic acid with ascorbic acid. Other disadvantage of acetic acid is that it has corrosive nature towards the mucus and the skin.

For the standardization of chitosan and its conjugated nanoparticles various concentrations of chitosan and glucose (0.5, 1, 1.5, 2.5, 3.5 and 5 mg/ml) with ascorbic acid (0.5, 1, 2 %) and TPP (1, 2, 3, 4 and 5 mg/ml) were prepared. Among them 2.5 mg/ml concentration of chitosan and glucose with 1 % ascorbic acid and 2 mg/ml of TPP gave good yield of nanoparticles with respect to size and charge of the synthesised nanoparticles. Care was taken while adding TPP, as TPP in higher concentration resulted in larger particle size. The formation of large size particle may be due to aggregation of the nanoparticle due to cross linking with each other.

4.2. Size and Zeta potential studies

The world's most widely used system. It is used for the measurement of the size, electrophoretic mobility of proteins, zeta potential of colloids and nanoparticles, and optionally the measurement of protein mobility and microrheology of protein and polymer solutions.Dynamic light scattering (DLS) studies were conducted to investigate the hydrodynamic size, polydispersity index and surface zeta potential of the conjugated chitosan nanoparticles in a colloidal aqueous environment. The main principle of DLS study is that when particles were dispersed in a medium the particles undergo Brownian motion in the suspended liquid solution which measures the fluctuations in the intensity of scattering light.Fig 4.2a shows the size possessed by the nanoparticles.The lowest size was observed in case of chitosan-HRS-AgNP of size 100±2,Cs-PEG of size 120±4 then comes the formulation Cs-AA of size 140±3. Vitamin E (α-tocopherol) is very viscous in nature the

conjugated nanoparticles were primarily colloidal in nature which is clearly observed in the DLS measurement (229.9±13 nm) as well as in Fe-SEM micrographs. The chitosan-catechol and chitosan-glucose-AA conjugated formulations were also found out to be in the nanorange having 155.9±20 nm and 182.8±5 nm respectively.The zeta potential deals with the charge of the nanoparticle.Fig 1b shows the charge possessed by the nanoparticle.It was found that all the formulation had positive charge.This positive charge on chitosan formulation have a very good significance as this can be easily taken up by the cancer cells.

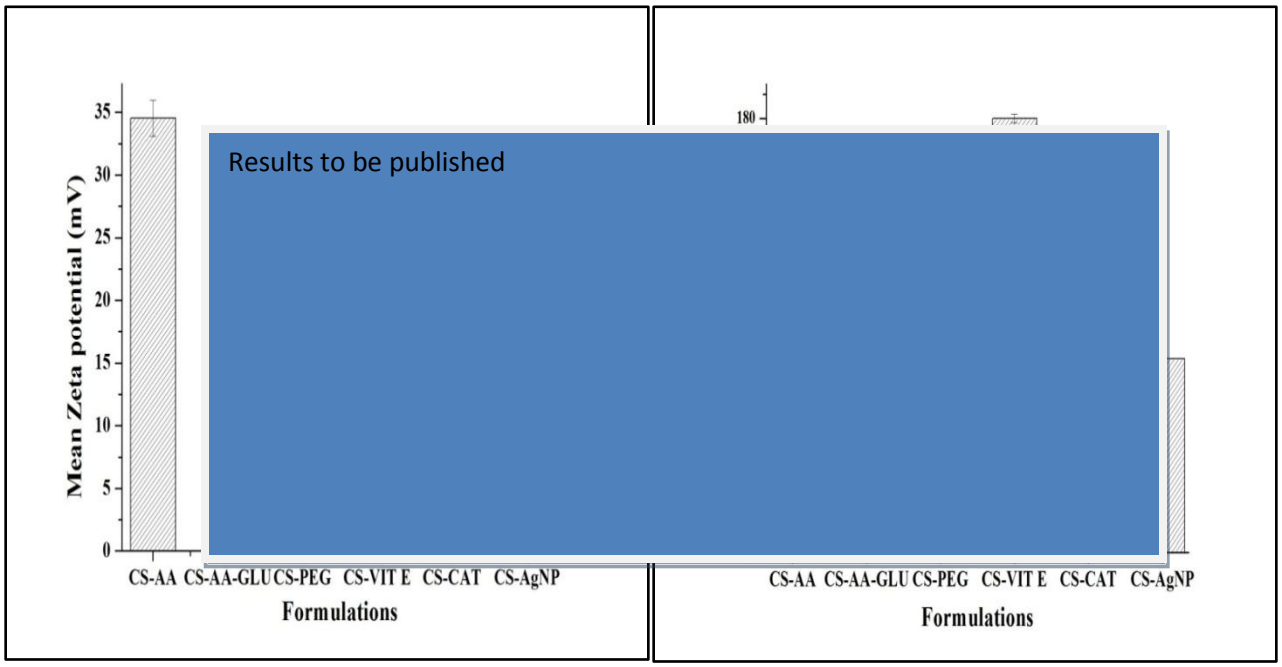


Fig4.2aFig4.2b

Fig4.2a:Graph depicts the mean size possessed bythe chitosan nanoparticle formulations. Fig4.2b:Graph shows the mean zeta potential of the nanoparticles.

4.3. Morphological studies by Fe-SEM

Figure given belowshows the surface morphology of the prepared chitosan based conjugated formulated nanoparticles by Field emission scanning electron microscopy (Fe-SEM). The average size of the particles were around 30-80 nm but in case of chitosan-Vit E conjugate the particles were more towards the emulsion type rather than in particle form which may be due to the viscosityassociated with VitaminE.

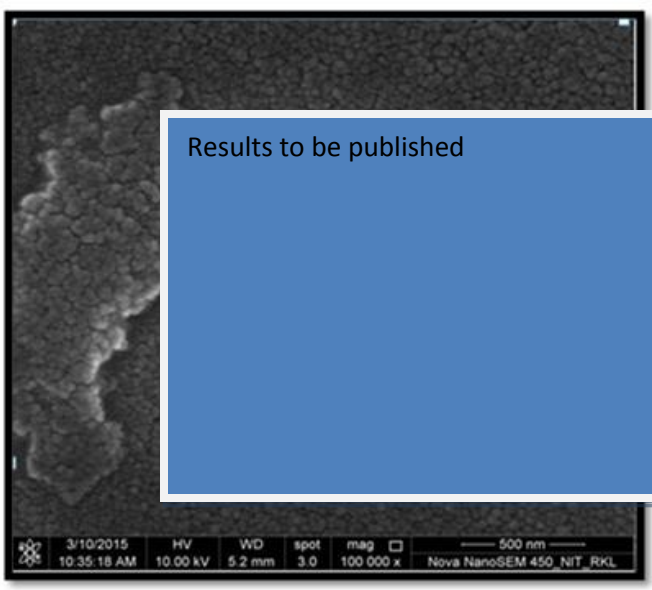


Fig 4.3a:Cs-AA

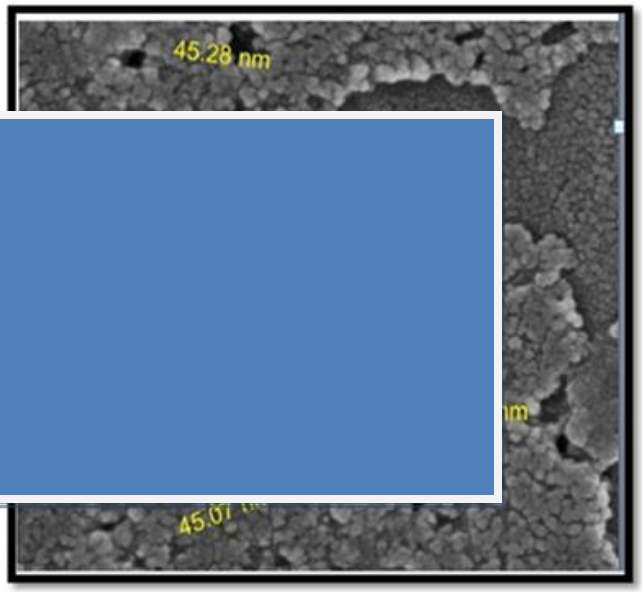


Fig 4.3b: Cs-AA-Glu

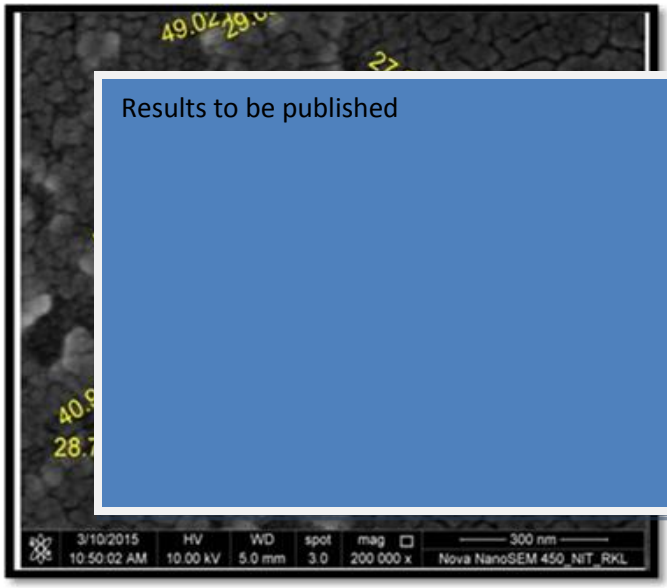


Fig 4.3c:Cs-Cat

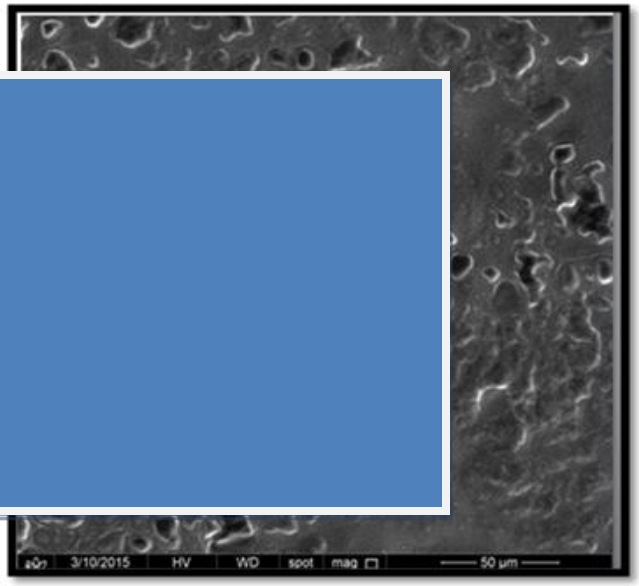


Fig 4.3d:Cs-Vit E

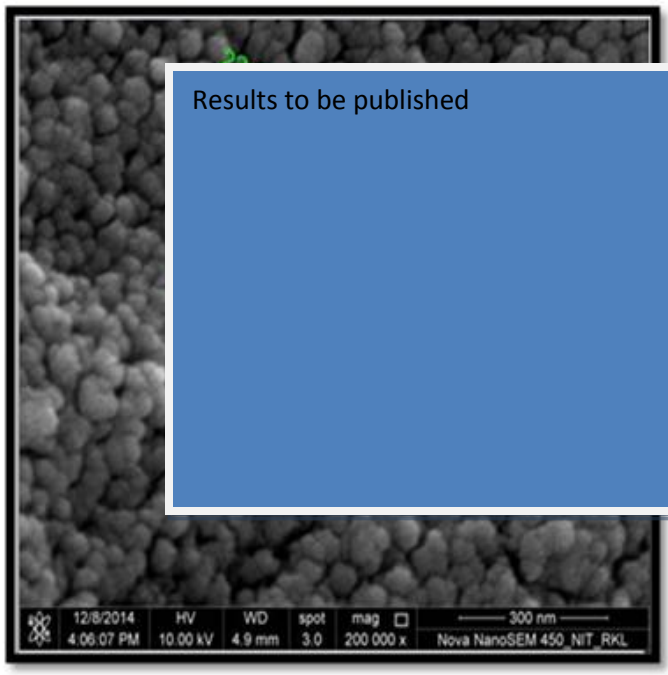


Fig4.3e:Cs-AgNP

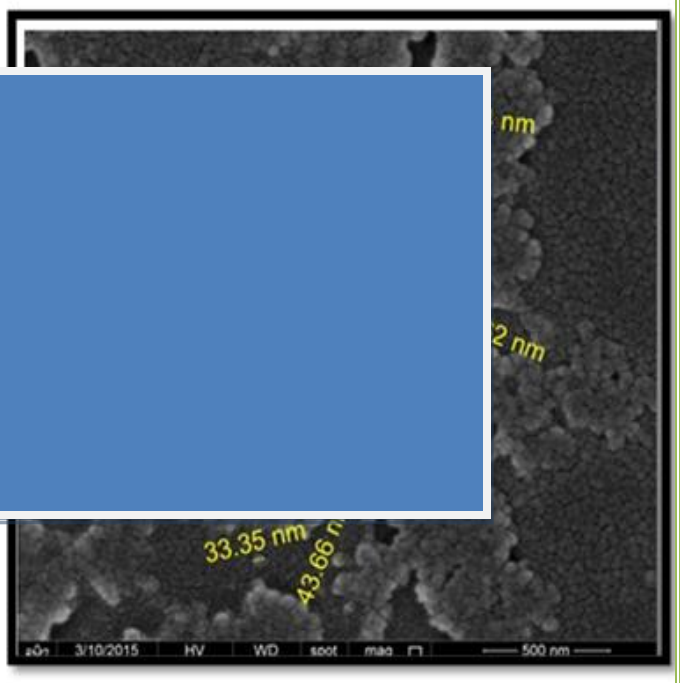


Fig 4.3f: Cs-PEG

4.4. The Attenuated Total Reflection Fourier Transform Infrared (ATR- FTIR)

The ATR-FTIR measurements were carried out to identify any modification that has occurred during the chemical transformation between chitosan, glucose, ascorbic acid, Vitamin E, catechol and silver Nanoparticles. In the chitosan-ascorbic acid-glucose conjugate spectrum strong bands were observed at 3744 (N-H stretching), 2924 (C-H stretching), 2536 (O-H stretching), 1562 (C=C bending) and 1097 (C-N stretching) cm^{-1}. Similarly common bands at 3740, 2912, 1096 cm^{-1} were observed for chitosan-catechol conjugate and chitosan-HRS-AgNPs conjugated nanoparticles. These same bands were also observed when pure chitosan, glucose, ascorbic acid, PEG, Vit E and silver nanoparticles were scanned in the 4000-400 cm^{-1} range. When both the conjugated and the standards were plotted side by side the chemical modifications were clearly visible in the ATR-FTIR spectrum.

4.5. X-ray powder diffraction (XRD)

XRD patterns of the conjugated chitosan nanoparticles with PEG,Vit E, catechol and HRS-AgNPs. Fig4.5a. shows sharp and intense diffraction peaks were observed for ascorbic acid , glucose, catechol , chitosan and HRS-AgNPs showed crystalline nature in its natural form. But after conjugation the nanoparticles showed no characteristic crystalline peaks of the individual components when scanned with same 2θ degrees, clearly elucidating their complete encapsulation with chitosan-ascorbic acid-glucose matrix. Fig4.5b shows the amorphous nature of the nanoparticles conjugated.

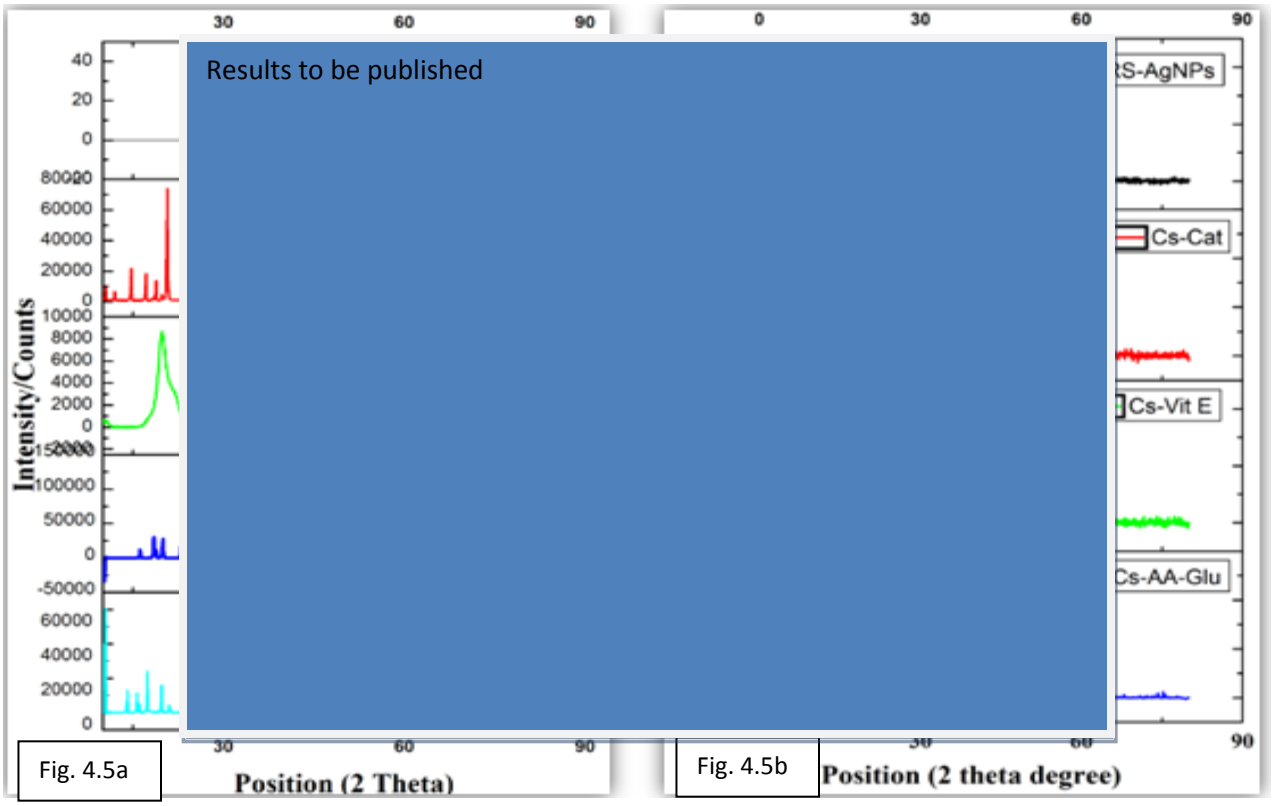


Fig. 4.5a

Fig. 4.5b

Fig 4.5a: Showing the crystalline nature of ascorbic acid,glucose,chitosan,glucose, catechol and silver Nanoparticles; **Fig 4.5b**showing the amorphous nature of chitosan- silvernanoparticle,chitosan-catechol,chitosan-vitamin e and chitosan-ascorbic acid-glucose conjugates.

4.6. Encapsulation efficiency (EE)

The % EE was found out to be 80.48±3.7 % for Cs-Vit E, 86.21±1.07% for Cs-Cat ,73.4±1.2% for Cs-Peg,75.2±3% for Cs-glu,76.3±2% Cs-Aaand 88.02±2.8% for Cs-HRS-AgNPs conjugated nanoparticles.

Table 1

Sl.No.	Sample	Encapsulation
1.	Cs- AA	76.3±2%
2.	Cs –AA-GLU	
3.	Cs-AA-GLU-PEG	
4.	Cs-AA-GLU-VIT E	
5.	Cs-AA-Glu-Cat	
6.	Cs-AA-AgNP	

Results to be published

Table 1 showing the percentage of drug encapsulated

4.7. Effect of pH on size and charge

pH plays a very crucial role during the preparation of a colloidal formulations. In the present investigation the blank and conjugated chitosan nanoparticles were subjected to various pH ranges (2, 4, 6, 8, 10 and 12).Fig 4.7ashows the effect of pH on the size of the prepared conjugated chitosannanoparticles. At acidic pH the size of the conjugated chitosan formulations were within the nanorange of 170-250 nm. With subsequent increase in pH towards the basic end the size of the formulations also increases where at pH 12 the formulations were of micro meter range. This is mainly due to the aggregation of the particles with increase in the pH values. Fig 4.7b shows the effect of pH on the surface zeta potential of the prepared chitosan-conjugated formulations. The

change of pH toward basic end reduced the zeta potential of nanoparticles towards negative value. The positive charge of the particles is generally due to the due to the ionization of amine groups in the chitosan molecules.

Fig 4.7a

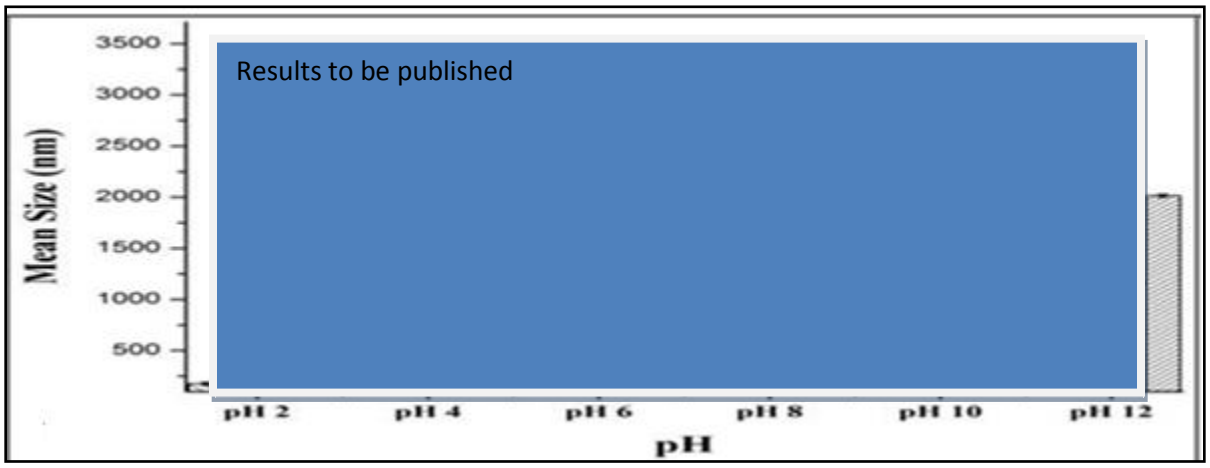


Fig 4.7b

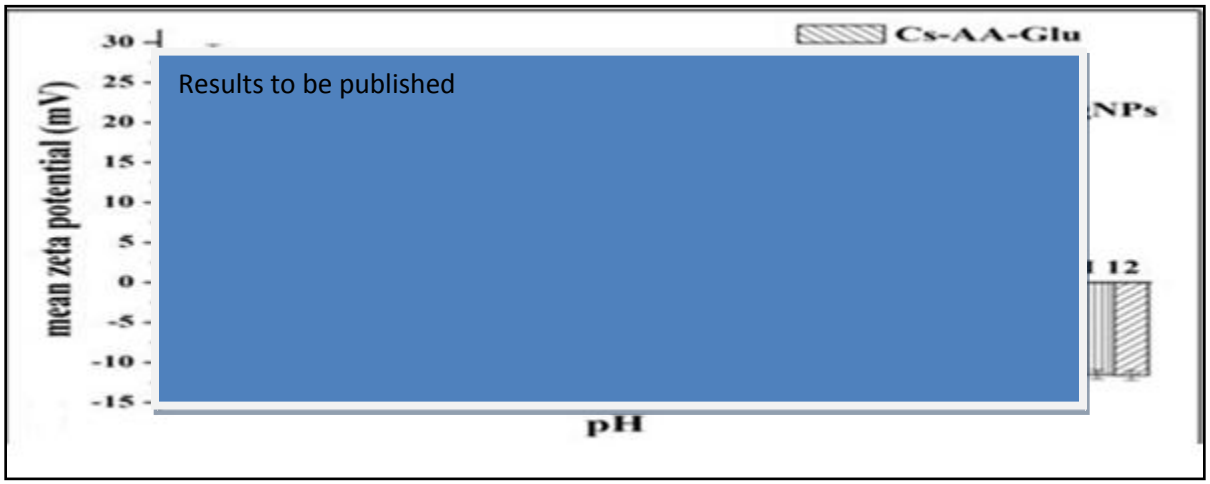


Fig 4.7a shows the effect of pH on mean size of Nanoparticles, whereas **Fig 4.7b**shows the effect of pH on mean zeta potential.

4.8.Antioxidant potential study

DPPH assay is a radical scavenging of 1,1-diphenyl-picryl-hydrazyl stable at room temperature which when dissolved in methanol gives a violet color. When DPPH comes in contact with an antioxidant compound it is reduced by donating hydrogen or free electrons .Thus the degree of decolourization confer the scavenging activity of the conjugated nanoparticles. Fig.4.8a clearly shows the DPPH scavenging potentials of the conjugated chitosan formulations in the following order of magnitude: Cs-AA-Glu> Cs-Cat > Cs-HRS-AgNPs> Cs-Vit E> Cs-AA > Cs-AA-Peg. Vitamin E is a known antioxidant but shows very less DPPH scavenging which may be due to its very less encapsulation with the chitosan base.

Fig 4.8b shows the hydrogen peroxide scavenging activity of the prepared chitosan based conjugated formulations. The graph clearly illustrates the H2O2 scavenging potentials of the six conjugated nanoparticles but Cs-Vit E nanoparticle showed a different scavenging potential where as the other formulations showed overall same type of scavenging activity with increase in concentration of the formulations. H_2O_2 is a weak oxidating agent which in itself is not very reactive but as it can cross cell membrane rapidly it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of its toxic effects.

Nitric oxide (NO) is one of the components of the ROS family and is implicated in various inflammation reactions, cancer and other pathological conditions. Fig4.8c shows the dose dependent scavenging activity of the six chitosan based conjugated nanoparticles where all the six chitosan conjugated formulations exhibited very excellent NO scavenging activity in the following order:, Cs-HRS-AgNPs> Cs-Cat > Cs-Vit E >Cs-AA-Glu>Cs-AA-Glu> Cs-AA-PEG.

Fig4.8d shows the ferrous reducing antioxidant power activity of the conjugated nanoparticles. From the graph it can be clearly illustrated that with increase in concentration there was increase in absorbance at 700 nm. All the conjugated nanoparticles show much or less the same pattern towards

scavenging of NO. The transition metal ion $Fe2^{+}$ possesses the ability to propagate the formation of free radicals by gain or loss of free electrons. Most ROS are generated as by products during mitochondrial electron transport and other metabolic reaction. In addition, ROS are formed as necessary intermediates of metal catalyzed oxidation reaction. Excess of metal ions can lead to various anomalies in the body. Thus the ability of the prepared nanoparticle for the chelating of $Fe2^{+}$ was calculated to be in order Cs-Vit E > Cs-AA-Glu> Cs-Cat > Cs- AA>Cs-HRS-AgNPs>Cs-AA-Peg .

Fig.4.8

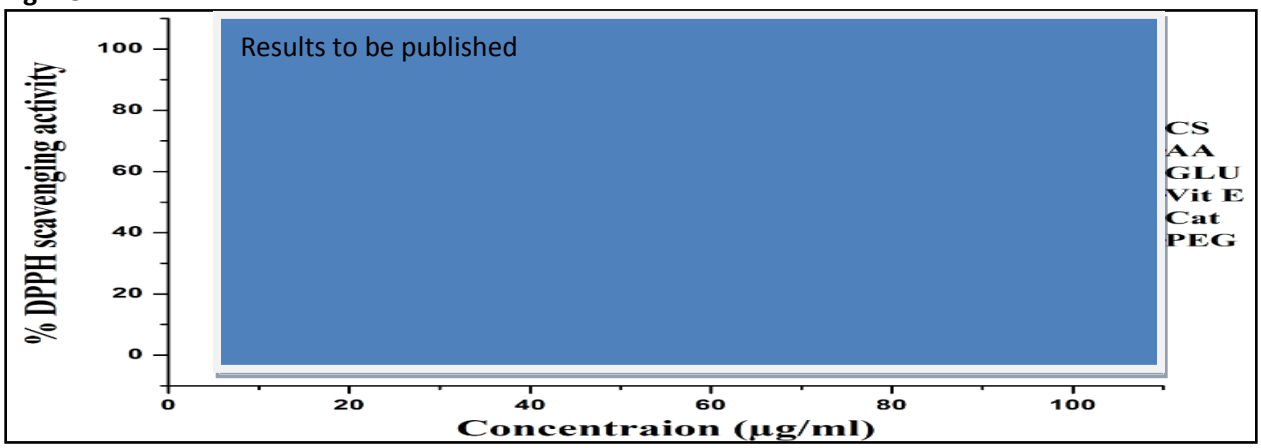


Fig. 4.8a

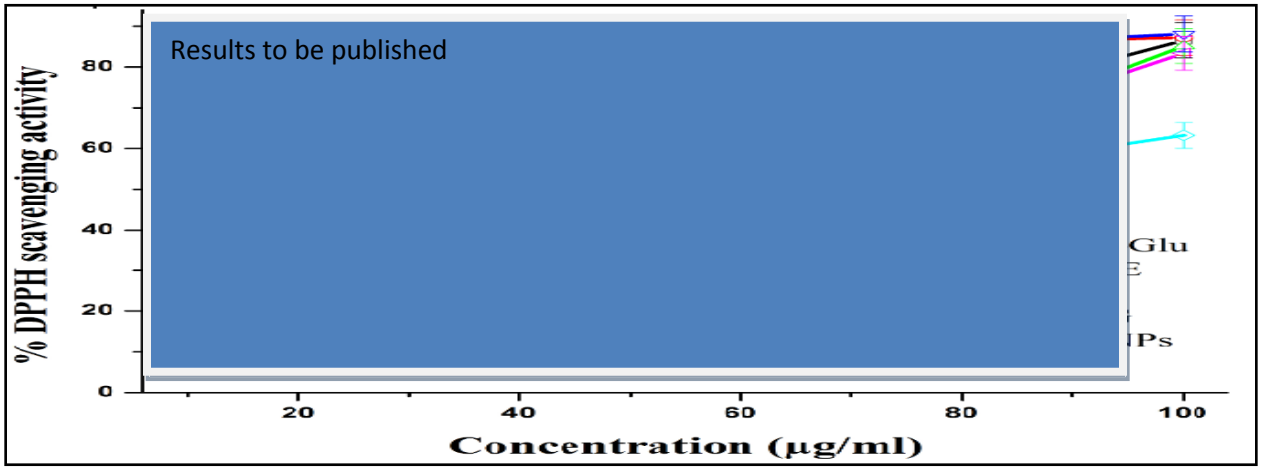
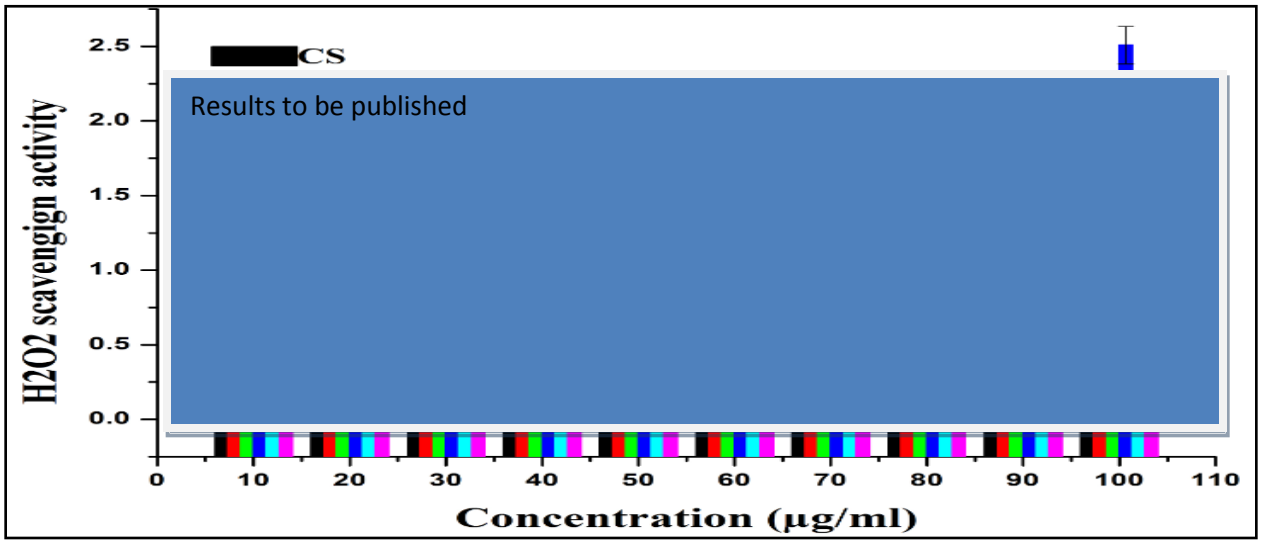


Fig 4.8 Shows the DDPH scavenging activity of the individual components used for nanoparticles conjugate synthesis andFig. 4.8a shows the DDPH scavenging activity of the conjugated nanoparticles

Fig. 4.8b



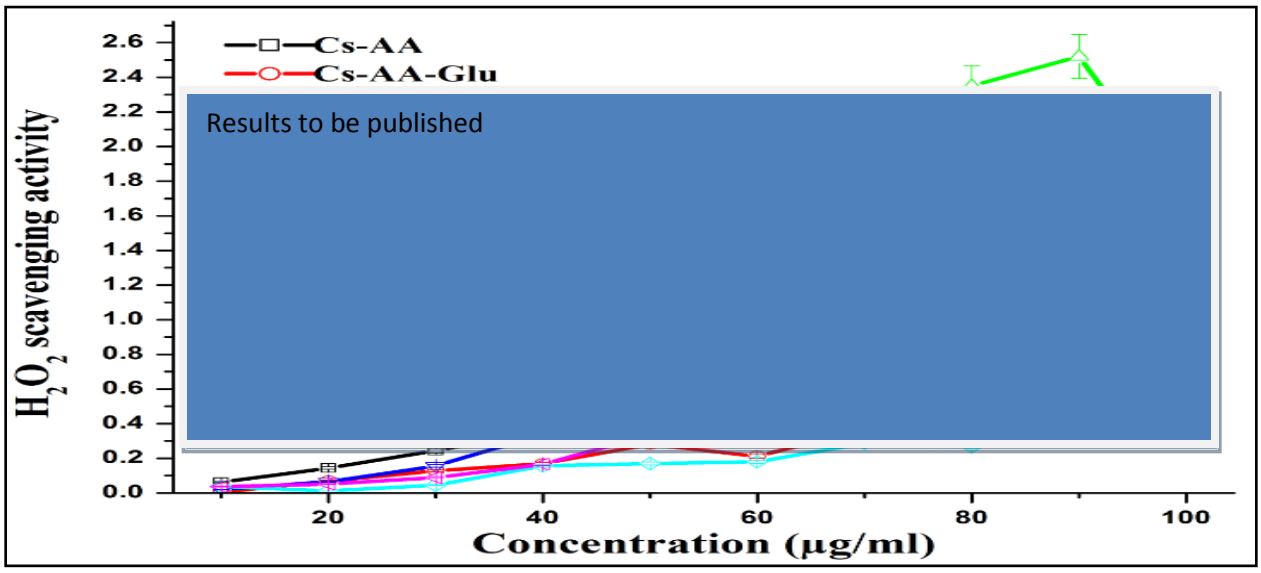
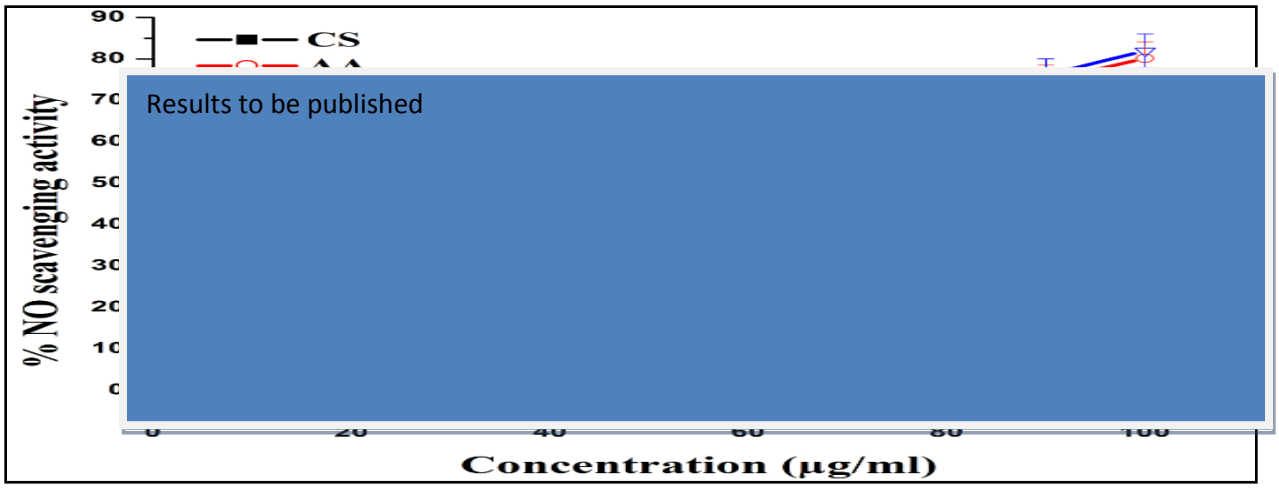


Fig 4.8c


CS
% NO scavenging activity
Concentration (µg/ml)
Results to be published

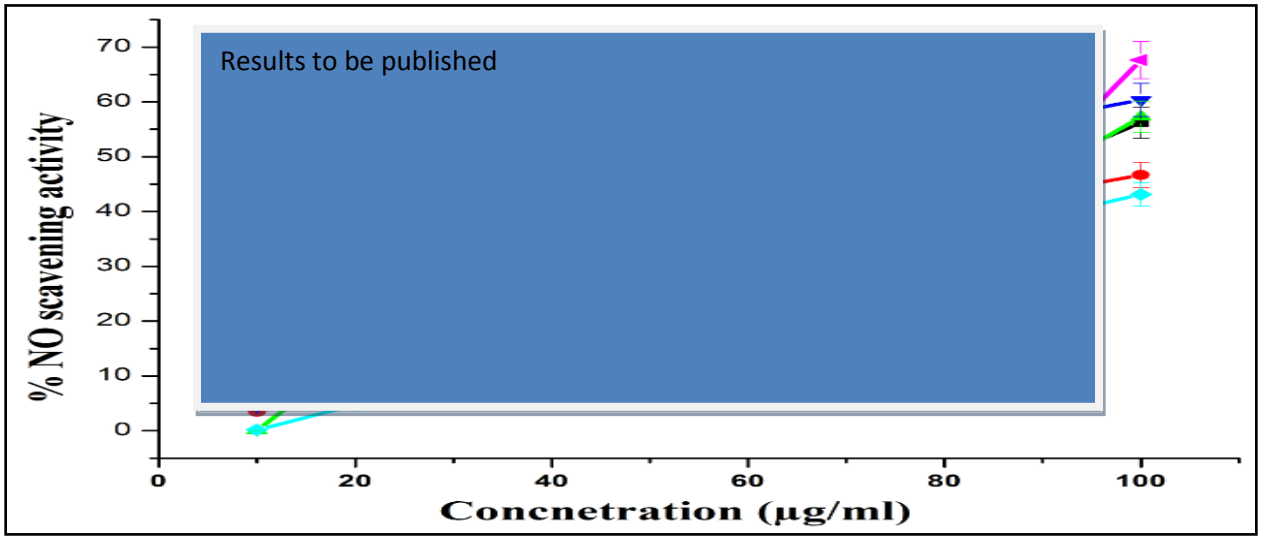
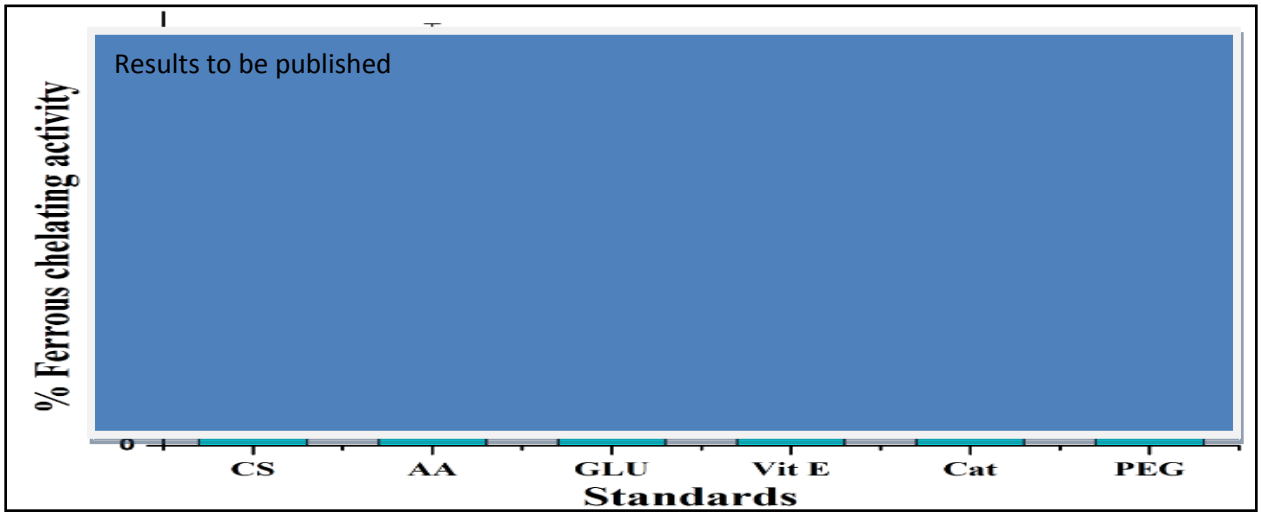

% NO scavening activity
Concnetration (µg/ml)
Results to be published

Fig 4.8d


% Ferrous chelating activity
CS
AA
GLU
Vit E
Cat
PEG
Standards
Results to be published

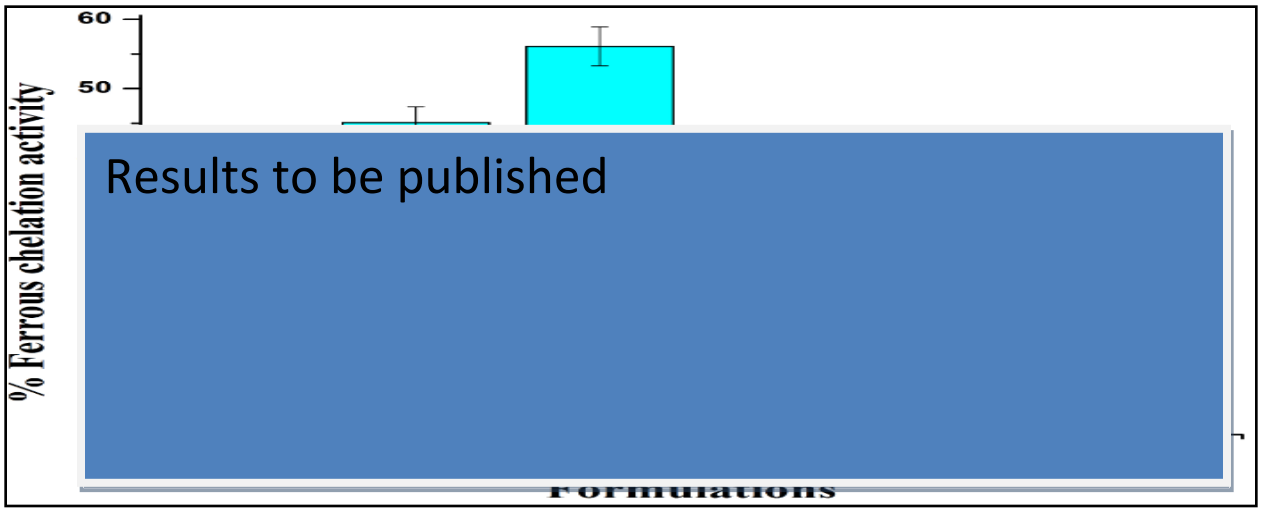
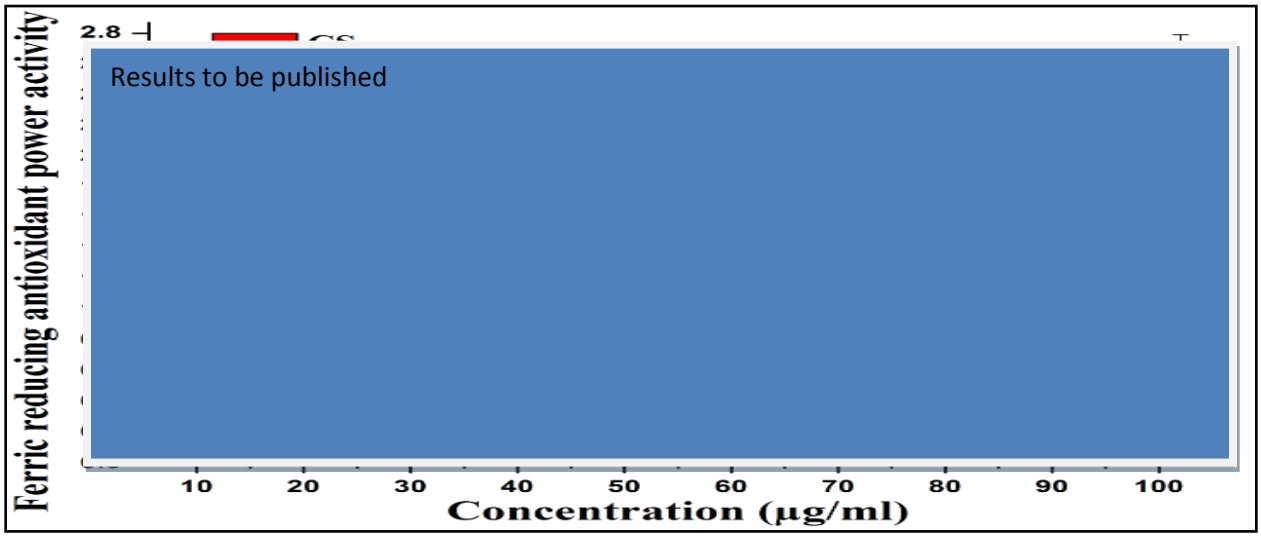
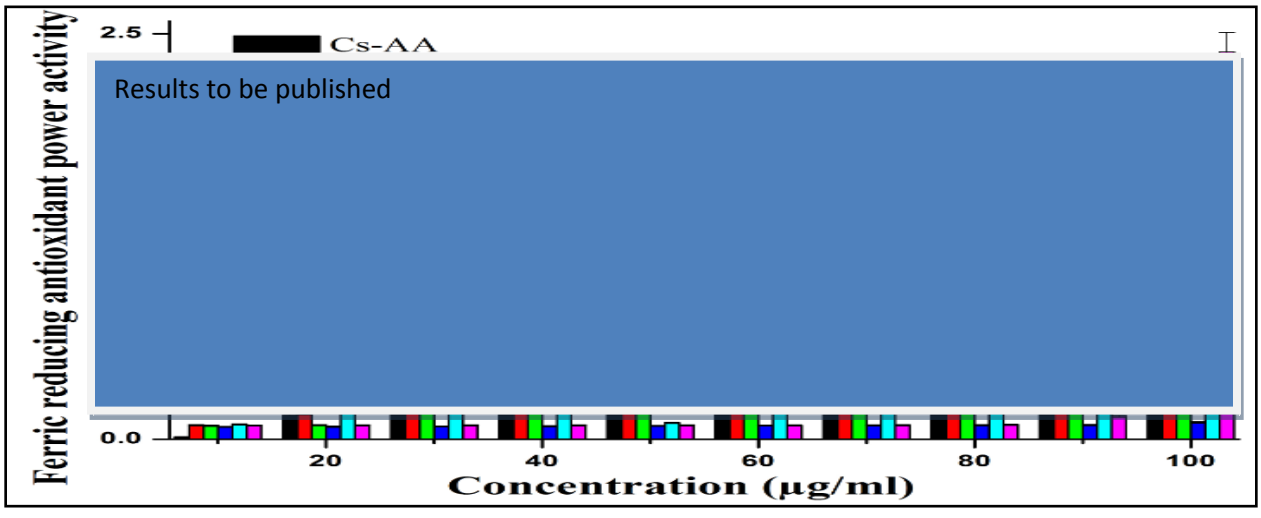

60
50
% Ferrous chelation activity
Results to be published
Formulations

Fig 4.8 e


2.8
Ferric reducing antioxidant power activity
Results to be published
10
20
30
40
50
60
70
80
90
100
Concentration (µg/ml)


2.5
Cs-AA
Ferric reducing antioxidant power activity
Results to be published
0.0
20
40
60
80
100
Concentration (µg/ml)

4.9.*In vitro* drug release study

Fig.4.9a shows the *in vitro* release of our drugs i.e., ascorbic acid, vitamin E, catechol and HRS-AgNPs. The release profile of ascorbic acid was very slow but it gained some momentum after 2 hours and upon reaching 12 hours of incubation almost 50% of ascorbic acid was released. In case of Vitamin E within 15 minutes of incubation has lead to 20% of its release and after 12 hours almost 83% of vitamin E has been released from the chitosan conjugated Vit. E nanoparticles. catechol also showed the same release pattern which was 62% upto 5 hours and after that there was very slow release profile but again after 10^{th} hour of incubation the release of catechol was at its maximum. The most slow release profile was by the HRS-AgNPs conjugated chitosan nanoparticles which were very slow upto 8 hours of incubation and after 12 hour it was 88% thus confirming its good encapsulation within the chitosan-TPP matrix. This type of slow release profile is very useful for targeted drug delivery where very slowly the drug is released in a uniform manner.

Fig4.9a

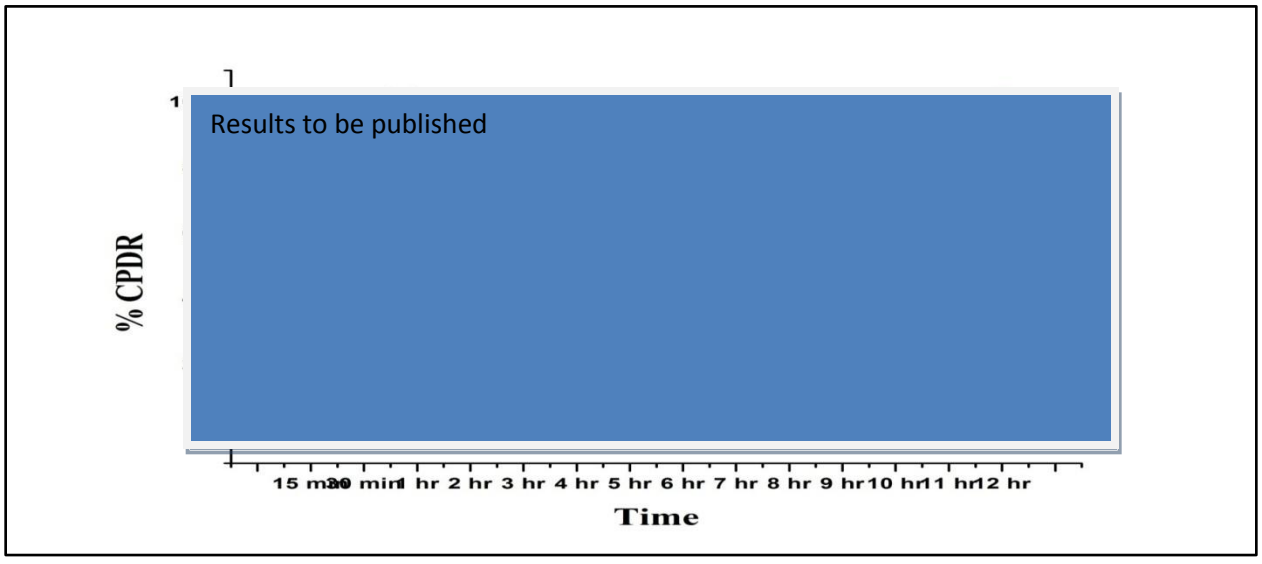


Fig 4.9a Showing the linear drug release for different molecules conjugated during the nanoparticles formation

4.10.Anticancer activity of the prepared conjugated nanoparticles

The main methods used for treatment of breast cancer are surgery, chemotherapy and radiotherapy. As after surgery there remains a prolong period of routine chemotherapy and radiotherapy so more targeted drug delivery routes needs to be developed. The inhibition of the cells (%) treated with various concentrations of conjugated chitosan nanoparticles are shown in fig 4.10a. The conjugated nanoparticles showed dose dependent toxicity towards MCF-7 breast cancer cell lines.With increase in concentration of the drug the viability of the cells decreases. The anticancer activity of formulated nanoparticle are in the order;Cs-AA-Glu> Cs-Vit E > Cs-Cat > Cs-HRS-AgNPs.

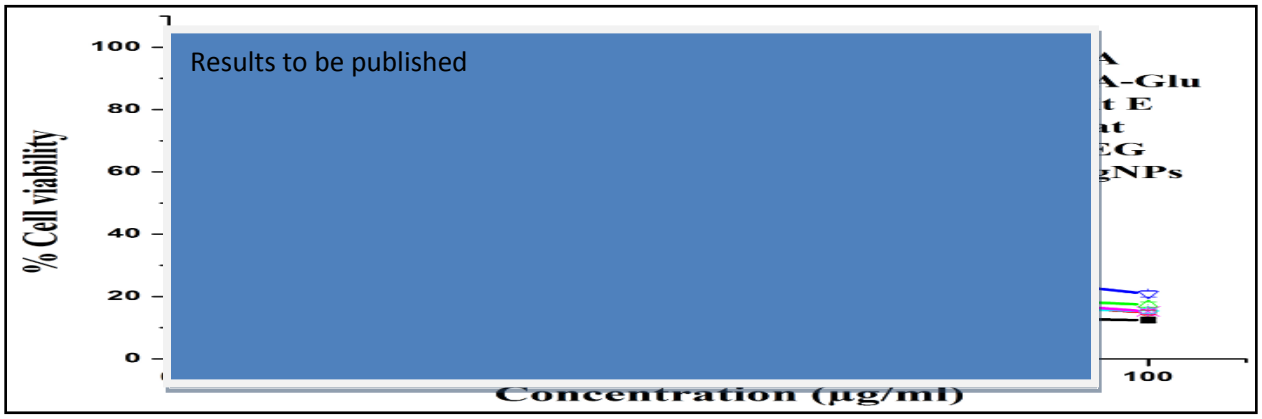


Fig. 4.10a showing the effect of drug when incubated with MCF-7 cell line .it predicts that with increase in drug concentration the cell viability decreases.

CONCLUSION

The current investigation was undertaken to develop chitosan based conjugated nanoparticles to deliver antioxidants to the targeted sites though the GLUT receptor pathways.The results obtained are of relevant importance in biomedical or biological sciences.The size and the charge possessed by chitosan based nanoparticles are with the range of drug delivery system to the cells.The charge of all the chitosan based formulation were found to be positive and the mean size ranges in between 50-200nm because of this possessed size and charge the nanoparticle can be easily taken up by the cancer cells.

The drug encapsulation percentage of all the nanoparticles are above 70%.In which CS-AgNP have the highest drug encapsulation percentage around 80% and CS-Cat around 80%. Drug release study showed the linear release of drug in PBS. The addition of PEG as drug conjugate enhances the drug retention and slower release of drug into PBS.

Coming to its antioxidant property it was observed that when individual component are treated to DPPH,Nitric Oxide or Hydrogen Peroxide they have relatively lower antioxidant property but in conjugated form they show increase in antioxidant property.

Further molecular level work has to be done before going for the *in vivo* studies.

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